WO 00/52156 PCT/AU00/00143

13/pp

REGULATORY CONSTRUCTS COMPRISING INTRON 3 OF PROSTATE SPECIFIC MEMBRANE ANTIGEN GENE

FIELD OF THE INVENTION

The present invention relates to constructs comprising a novel regulatory element derived from a prostate specific gene. The present invention also relates to diagnostic and therapeutic methods involving the use of these constructs.

BACKGROUND OF THE INVENTION

The isolation and characterisation of DNA regions which control tissue specific and/or hormonally-regulated gene expression has been an important to the understanding of the developmental processes by which expression of particular genes is limited to specific cell types. Promoter regions are found immediately upstream and often overlapping the start site(s) of transcription and are critical for initiation and basal levels of transcription. Enhancers are regulatory regions which may lie some distance from the transcription start site, either upstream or downstream of a gene or within introns and which often confer high level tissue specific or hormonally-regulated expression; in some cases their action is specific to particular promoters. The function of both promoters and enhancers is mediated by specific proteins, transcription factors, that bind to specific DNA sequences. Alone or in combination with other transcription factors they recruit the core transcription machinery including RNA polymerase to the transcription initiation site and act to stimulate their activity. Isolated promoters and enhancer sequences can be used, in gene therapy for example, to direct expression of other genes in a cell or tissue specific manner and also provide targets for the development of agents that can specifically modulate gene expression.

The promoters and regulatory regions of a number of genes that are expressed in the prostate have been studied either using transfection techniques or by following gene expression in transgenic mice. We have previously compared the cell-type specificity of expression directed by promoters of the prostate-expressed genes, probasin (Pb) and relaxin genes and the promoter and enhancer of the prostate specific antigen (PSA) gene (1). Most of the genes identified as prostate-specific are androgen-inducible and this aspect of their function has been studied in some detail. Thus the importance

of androgen response elements for induced expression and/or binding of androgen receptor have been characterised in the PSA (2, 3), human glandular kallikrein (KLK2) (4), rat prostatic steroid binding proteins (PSBP) (5, 6), probasin Pb (7, 8) and prostatic acid phosphatase genes (9) and in regulatory elements in the introns of the rat PSBP C3(1) gene (10) and the rat 20-KDa androgen regulated protein (11).

Among the core promoter regions analysed only that of the probasin gene confers substantial prostate specificity of expression (1, 15). Elements involved in conferring prostate-specificity of expression per se, as distinct from androgen responsiveness, have not been well characterised, though tissue-specific factors binding to regions of the PSBP C3 gene promoter and 1st intron have been identified (9, 12). The gene for rat PSBP C(3) with 4 kb upstream and 2 kb downstream flanking sequences is expressed tissue-specifically and with appropriate hormonal control in transgenic mice (13). The use of a 5kb upstream region from the rat PSBP C3(1) gene to express the SV40 T-antigen could elicit prostate tumours, but expression was not highly restricted and other abnormalities were common (14). Studies with transgenic mice have established that regions of the probasin and PSBP C(3) genes can confer prostate specificity.

The PSA and probasin regulatory regions are the two most studied among prostate-expressed genes. It has been established that a 430 bp region upstream of the rat probasin gene is able to confer prostate specificity of expression on reporter genes in transfection experiments (1) and in transgenic animals (15, 16); when used to target expression of the SV40 T-antigen, prostate tumours develop specifically (17, 18). This expression is not totally specific but specificity is significantly improved by the inclusion of MAR (matrix attachment regions) from the chick lysozyme gene (15). The 430 bp promoter region is strongly responsive to androgen induction and androgen response elements which bind the androgen receptor (AR) have been characterised (4, 6, 7, 16).

The PSA upstream region (to -630 bp) also acts as a strongly androgen responsive promoter and androgen response elements have also been characterised (2, 3). However, this region is not sufficient to direct cell type specific expression in culture (1) or tissue specific expression in transgenic mice (19). Use of the 630 bp human PSA promoter region to express an activated Ha-ras oncogene in transgenic mice led to the development of salivary

gland and not prostate tumours (19). Pang et al. have reported that the equivalent promoter region isolated from a prostate cancer patient contained 7 mutations compared to the published sequence and was highly active in the prostate cancer cell line LNCaP (20, 21). More recently, an enhancer region has been identified in the region 4 to 5kb upstream of the transcription start site of the PSA gene (20, 21). This PSA enhancer has been shown to act as an androgen-inducible enhancer and in combination with the PSA promoter to display significant cell-type specificity (1, 20, 21).

Prostate-Specific Membrane Antigen

Prostate specific membrane antigen (PSMA) is one of the few prostate-specific proteins identified whose expression is not induced by androgens. PSMA was first identified as the antigen bound to by the monoclonal antibody 7E11-C5(25). The antibody was raised against a membrane fraction of the prostate cancer cell line LNCaP and was shown to bind specifically to normal prostate tissue as well as primary and metastatic prostate cancer tissue. This antibody was later found to bind to an internal epitope of this membrane-bound protein (26, 27). Subsequently, other monoclonal antibodies targeted to the extracellular domain of the protein have been isolated (28, 29).

The cDNA encoding PSMA has been cloned and its sequence determined (30). PSMA is a Type II integral membrane protein and is associated with the plasma membrane of expressing cells such as LNCaP (30). A splice variant of PSMA (Psm') that lacks the membrane anchor domain and has been shown to be cytoplasmically located has also been identified (31) The ratio of PSMA to Psm' has been reported to be increased in prostate cancer as compared with normal prostate or benign hyperplasia (31). PSMA has been shown to possess two related enzymatic activities, it acts as a carboxypeptidase (folate hydrolase) on poly γ-glutamated folates (32) and as a peptidase on the acidic neuropeptide N-acetylaspartyl glutamate (33). This latter activity is consistent with the expression of PSMA or a related protein in the brain

The specificity of PSMA expression has been studied at both the protein and RNA level. In addition to its major site of expression in the prostate immunohistochemical studies have identified PSMA expression in the duodenum brush border/small intestine, in a subset of proximal tubules in the kidney and in rare cells in the colon (34, 35). All other normal issues studies have been negative for expression, except for striated muscle which stains with

the 7E11-C5 antibody, but not with antibodies to the external domain of PSMA (28).

Both the 7E11-C5 and external domain antibodies have been found to react with tumour vasculature of a wide range of human tumour types (28, 36), indicating specific induction of PSMA expression. PSMA expression has not been identified in any normal vasculature.

RNA expression has been found to largely parallel the protein expression data. RNAse protection analysis identified PSMA mRNA in the prostate, salivary gland and brain and sometimes in the small intestine (37). The identification of PSMA RNA in the brain is consistent with the cloning of a closely related cDNA from rat brain (33). Immunohistochemical analyses have failed, however, to identify antigenically reactive PSMA in human brain tissue.

PSMA expression has been shown to be down regulated in the presence of androgens and expression is generally elevated in late stage prostate cancer and in patients undergoing androgen deprivation or ablation therapies (37, 38). Expression of PSMA has also been found to be regulated by a number of growth factors: bFGF, TGF-α and EGF upregulate expression while TNF-α decreases it (39).

The restricted high level expression of PSMA in prostate cells and the induction of its expression in the vasculature of a wide range of tumours make it ideal for the targeting of prostate and other tumour types. Genomic clones encompassing the PSMA gene have been isolated and its sequence and exon/intron structure determined (40). Regulatory regions controlling its expression may find use in gene therapeutic cancer treatments, enabling the restricted or high level expression in the target cell types. Such regulatory regions also provide a target for the development of agents that may interfere with gene expression in the target cell types.

SUMMARY OF THE INVENTION

The present inventors have identified a novel regulatory element in the PSM gene. The regultory element is an enhancer located in intron 3 of the PSM gene. This is the first report, to the applicant's knowledge, of an enhancer derived from an androgen-independent, prostate specific gene

When used herein, the PSM gene refers to the PSM genomic sequence described in *O'Keefe et al, 1998* (40) (Genbank accession number AF007544), the entire contents of which are incorporated herein by reference.

Accordingly, in a first aspect the present invention provides a recombinant polynucleotide comprising at least one regulatory element derived from intron 3 of the PSM gene and a sequence encoding a heterologous polypeptide.

By "heterologous polypeptide" we mean a polypeptide other than the prostate specific membrane antigen (PSMA) polypeptide.

In a preferred embodiment, the recombinant DNA molecule further comprises a promoter. Preferably, the promoter is located upstream from and is operably linked to the sequence encoding the polypeptide.

In a second aspect, the present invention provides a recombinant expression cassette comprising at least one regulatory element derived from intron 3 of the PSM gene, a promoter, and an insertion site into which a coding sequence is optionally inserted, the insertion site being adjacent to and downstream of the promoter.

The regulatory element(s) may be located in either orientation anywhere within the recombinant DNA molecule or expression cassette of the present invention. For example, the regulatory element may be located downstream of the coding sequence (eg. downstream of the 3' termination or polyadenylation signals) or within an intron located in the coding sequence. In a preferred embodiment, the regulatory element is located adjacent to the promoter. More preferably, the regulatory element is upstream of the promoter.

Within the context of the present invention, it is preferred that the regulatory element is an enhancer element. Preferably, the enhancer element includes intron 3 of the PSM gene or a part thereof.

In a preferred embodiment, the enhancer element comprises

(a) a sequence comprising nucleotides 14,045 to 15,804, nucleotides 14,760 to 15,804, nucleotides 14,760 to 16,575 or nucleotides 14,045 to 16,575 of the PSM gene; or

(b) a nucleic acid sequence which hybridises under high stringency to a sequence defined in paragraph (a).

In one preferred embodiment, the enhancer element comprises nucleotides 14760 to 14930 as shown in Figure 11 or a sequence which hybridises thereto under high stringency.

In another preferred embodiment, the enhancer element comprises nucleotides 14760 to 15091 as shown in Figure 11 or a sequence which hybridises thereto under high stringency.

In a further preferred embodiment the recombinant DNA molecule or expression cassette of the present invention comprises two or more regulatory elements derived from intron 3 of the PSM gene. In one preferred embodiment, the recombinant DNA molecule or expression cassette comprises a dimer or higher multimer of regulatory elements derived from intron 3 of the PSMA gene.

It will be appreciated by those skilled in the art that any suitable promoter may be used in the context of the present invention. Preferred promoters include, but are not limited to, herpes virus thymidine kinase (TK), and Rous sarcoma virus (RSV) promoters, promoters active in the prostate, such as probasin, PSM, and PSA, or promoters active in vascular endothelium.

In a preferred embodiment, the recombinant DNA molecule and expression cassette of the present invention further comprise a polyadenylation signal located downstream from and operably linked to the sequence encoding the polypeptide, or downstream from the insertion site. Preferably, the polyadenylation signal is the SV40 polyadenylation signal or the bovine growth hormone polyadenylation signal as described in US 5122458, the entire contents of which are incorporated herein by reference.

In a third aspect, the present invention provides a vector comprising a recombinant DNA molecule of the first aspect or an expression cassette of the second aspect.

In one preferred embodiment the vector comprises a gene encoding a selectable marker. The vector may further include an origin of replication.

It is presently preferred that the vector is human adenovirus Type 5 or ovine adenovirus.

In a fourth aspect the present invention provides an isolated nucleic acid molecule, the nucleic acid molecule having enhancer activity and comprising

(a) a sequence comprising nucleotides 14760 to 14930 as shown in Figure 11, or

(b) a nucleic acid sequence which hybridises under high stringency to the sequence defined in paragraph (a).

In a preferred embodiment of the fourth aspect, the isolated nucleotide molecule comprises

(a) a sequence comprising nucleotides 14760 to 15091 as shown in Figure 11, or

(b) a nucleic acid sequence which hybridises under high stringency to the sequence defined in paragraph (a).

In a further preferred embodiment of the fourth aspect, the isolated nucleotide molecule is less than 7.5 kb.

In a fifth aspect the present invention provides a method for directing expression of a coding sequence of interest in a cell, the method comprising introducing into the cell a recombinant expression cassette comprising at least one regulatory element derived from intron 3 of the PSMA gene, a promoter, and a coding sequence, wherein the regulatory element and promoter direct expression of the coding sequence.

In a preferred embodiment of the fifth aspect, the cell is a prostate cell, bladder cell, breast cell or vascular endothelial cell.

In a sixth aspect the present invention provides a method for the treatment of cancer which method comprises administering to a subject a recombinant expression cassette comprising at least one regulatory element derived from intron 3 of the PSM gene, a promoter, and a coding sequence, wherein the regulatory element and promoter direct expression of the coding sequence.

In a preferred embodiment of the sixth aspect, the coding sequence encodes a toxin, a protein involved in viral replication, or an enzyme which converts a prodrug to a toxic drug. For example, the coding sequence may encode the enzyme purine nucleoside phosphorylase which converts the prodrugs fludarabine and 6-methylpurine 2-doexyriboside (6MPDR) to their toxic derivatives.

As the constructs of the present invention are useful for expression of proteins in vascular endothelial cells, a range of cancer types may be treated within the context of the sixth aspect of the present invention. Examples of suitable cancer types include renal cell carcinoma, transitional cell carcinoma

colonic adenocarcinoma, neuroendocrine carcinoma, malignant melanoma, pancreatic duct carcinoma, breast carcinoma, soft tissue carcinoma, non-small cell lung carcinoma, testicular embryonal carcinoma and glioblastoma multiforme. In a preferred embodiment of the sixth aspect, however, the cancer is selected from prostate, bladder or breast cancer.

As will be appreciated by those skilled in the field, the present invention provides novel regulatory elements from a gene expressed specifically in prostate, which are active both in the presence and absence of androgens. These regulatory elements may therefore be used for high level gene expression in prostate cells. Combinations of one or more of the regulatory elements with the probasin and PSA promoters are examples of constructs that provide for high level expression with strong prostate specificity.

The regulatory elements of the present invention may also be useful for directing expression in a limited range of other cell types, including tumour neovasculature and kidney cells.

The regulatory elements of the present invention may be used to target specific expression of genes to prostate cells or tumour neovasculature or kidney cells in gene therapy.

The regulatory elements of the present invention may also be used to target specific expression of genes in the development of transgenic animal models of prostate disease.

The regulatory elements of the present invention may also be used to identify other genetic elements which are involved in the regulation of gene expression in prostate cells.

The regulatory elements of the present invention may also be used in assays to identify reagents that interfere with prostate gene expression, or to identify proteins and other factors involved in regulation of prostate gene expression.

When used herein, "high stringency" refers to conditions that

(i) employ low ionic strength and high temperature for washing after hybridisation, for example, 0.1 x SSC and 0.1% (w/v) SDS at 50°C;

(ii) employ during hybridisation conditions such that the hybridisation temperature is ≤ 25°C lower than the duplex melting temperature of the hybridising polynucleotides, for example 1.5 x SSPE, 10% (w/v) polyethylene glycol 6000, 7% (w/v) SDS, 0.25 mg/ml fragmented herring sperm DNA at 65°C; or (iii) for example, 0.5M sodium phosphate, pH 7.2, 5mM EDTA, 7% (w/v)

SDS and 0.5% (w/v) BLOTTO at 70°C; or (iv) employ during hybridisation a denaturing agent such as formamide, for example, 50% (v/v) formamide with 5 x SSC, 50mM sodium phosphate (pH 6.5) and 5 x Denhardt's solution at 42°C; or (v) employ, for example, 50% (v/v) formamide, 5 x SSC, 50mM sodium phosphate (pH 6.8), 0.1% (w/v) sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg ml) and 10% dextran sulphate at 42°C.

Throughout this specification, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting Examples and Figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. pPSMentrap Vector. Key features of the vector are shown: the multicloning site (MCS) unique restriction sites upstream of the PSM 1k promoter region (PSM1k), leader sequence and intron (intron) derived from the pCI vector(Promega), the green fluorescent protein gene (GFP) and 3' sequences derived from the bovine growth hormone gene (bGHpA). A selection of useful restriction enzyme sites are shown: unique restriction enzyme sites are shown in bold.

Figure 2. Location of cloned PSM enhancer fragments: the map shows the location of the cloned enhancer fragments within intron 3 of the PSM gene. Base numbers (Genbank Accession No. AF007544) are indicated for the boundaries of intron 3 and for the ends of the cloned segments. The locations of the restriction sites SmaI (Sm), HinDIII (H) and SpeI (Sp) within the intron are shown. The arrows indicate the orientation of the cloned sequences within the pPSMentrap vector (see Figure 3). The right hand end of the enhancer clone #1 is shown as a stippled box since this end of the clone has undergone rearrangement: The SmaI, HinDIII and SpeI sites are present in all three cloned regions.

Figure 3. Promoter and enhancer inserts in pPSMentrap: The positions of the PSM 1kb promoter region and flanking restrictions sites in pPSMentrap are shown on the top line. To the right of the promoter sequences are the leader sequence and chimeric intron and GFP reporter gene. Below are shown maps of clones containing the En3 and En4 inserts. The sequences are in opposite orientation (note order of HinDIII and SpeI sites). Restriction sites are abbreviated as follows:

B2 BglII **E** EcoRI **H** HinDIII **K** KpnI **M** MfeI **N** NsiI
Nh NheI **P** PstI **S** SalI **Sp** SpeI **X** XbaI

Figure 4. Promoter and enhancer inserts in pCAT3SAT. Maps show the positions of the PSM 1 kb promoter, PSM En4 and the RSV promoter and their flanking restriction enzyme sites. To the right of the promoters is the leader

sequence and chimeric intron and CAT reporter gene as present in the Promega pCAT3 Basic vector. Restriction enzyme sites are abbreviated as follows:

B2 BglII	**Bz** BstZI	**E** EcoRI	**H** HinDIII	**K** KpnI	**M** MfeI
Ml MluI	**N** NsiI	**Nh** NheI	**P** PstI	**S** SalI	**Sc** SacI
Sm SmaI	**Sp** SpeI	**X** XbaI	**Xh** XhoI		

Figure 5. Relative CAT expression directed by the PSM Enhancer4/PSM1k promoter. Following transfection of pPSM1k-C3S or pEn4PSM1k-C3S into the cell lines indicated normalised expression levels were determined for each construct and are expressed relative to that determined from transfection of the pRSV-C3S plasmid.

Figure 6. Promoter and enhancer inserts in pGL3. Maps show the position and flanking restriction enzyme sites of the PSM 1kb promoter (shaded boxes), PSM enhancer fragments (solid boxes) and the RSV promoter (diagonal shading) in the different constructs prepared in the pGL3 vector. To the right of the region shown is the leader and chimeric intron and luciferase reporter gene of the pGL3 vector. PEN4PSM1k-GL3 and pEn3PSm1k-GL3 contain sequences of enhancer clones #4 and #3 respectively as shown in Figure 2. pEn3+4PSM1k-GL3 contains PSM enhancer sequences encompassing bases 14,045 to 16,575 (see Figure 2). POverlap3,4aPSM1k-GL3 and pOverlap3,4bPSM1k-GL3 contain enhancer sequences from bases 14,760 to 15,804, the a and b constructs containing the enhancer sequences in opposite orientations as indicated by the position of the HinDIII and SpeI sites. Restriction enzyme sites are abbreviated as follows:

A ApoI	**B2** BglII	**Bz** BstZI	**E** EcoRI	**Eo** EcoO109I	**H** HinDIII
K KpnI	**M** MfeI	**Ml** MluI	**N** NsiI	**Nh** NheI	**Nt** NotI
P PstI	**RV** EcoRV	**S** SalI	**Sc** SacI	**Sc2** SacII	**Sm** SmaI
Sp SpeI	**X** XbaI	**Xh** XhoI			

Figure 7. Relative luciferase expression of PSM enhancer/promoter constructs in the pGL3 vector. Mixtures of luciferase reporter plasmids (1.5 μg) and the normalising plasmid pRSV-CAT (1 μg) were transfected into different cell lines as shown. Normalised luciferase expression was determined and activity of the different plasmids expressed relative to the normalised expression from pRSV-GL3. Numbers above the columns indicate the relative

enhancement of activity compared with expression from the PSM promoter alone construct, pPSM1k-GL3.

Figure 8. PSM enhancer constructs with other promoters. Maps show the positions and flanking restriction enzyme sites of the PSM enhancer sequences (En4, solid boxes), and promoters from the PSA (diagonal pattern), probasin (vertical pattern) and thymidine kinase (horizontal pattern) genes. To the right of the promoters is the CAT reporter gene of the pCATSAT vector. Restriction enzyme sites are abbreviated as follows:

B BamHI **B2** BglII **E** EcoRI **H** HinDIII **N** NsiI **P** PstI
S SalI **Sm** SmaI **Sp** SpeI **X** XbaI

Figure 9. Relative enhancement of heterologous promoters by PSM En4.

a. Prostate cell lines
b. Non-prostate cell lines

The different promoter and enhancer constructs were transfected into cell lines as shown and CAT reporter gene expression normalised against SAT expression determined. Activities are expressed as a percentage of the normalised expression of pRSV-CAT. Numbers above the columns indicate the relative enhancement of activity compared with expression from the respective promoter alone constructs. An * indicates that expression levels were too low to determine a ratio.

Figure 10. Effect of androgen on enhancement of heterologous promoters by PSM En4. Plasmids containing the different enhancer/promoter combinations as indicated below the graph were transfected into LNCaP cells that were maintained in medium that had been charcoal stripped to remove androgens or in equivalent medium to which the non-metabolizable androgen analogue R1881 had been added to 0.28 nM. The presence or absence of androgen is also indicated (- or +) below the graph. Activities were determined and expressed as described in Figure 9.

Figure 11. Sequence of 331 base pair core region of the PSME (SEQ ID NO:1).

Figure 12. Specificity of purine nucleoside phosphorylase (PNP) gene expression in viral constructs OAV223 and OAV623 (PSME and probasin

promoter), OAV220 (PSME and RSV promoter) and OAV222 (PSME and CMV promoter).

EXPERIMENTAL DETAILS

Example 1

Isolation of PSMA gene enhancer sequences

Analyses of the region upstream and encompassing the transription start site of the PSMA gene (40) has shown that a 1kb region directs expression of reporter genes in the prostate cell line LNCaP. This expression shows specificity for prostate cells when compared to that directed by the SV40 enhancer/promoter. Expression in LNCaP cells was about 75% of that directed by the SV40 enhancer/promoter. Comparison with another widely expressed promoter, that of the Rous sarcoma virus (RSV) has indicated that the SV40 enhancer/promoter is only very weakly active, <1% of RSV activity, in LNCaP cells (unpublished data). We have cloned regions encompassing up to 11kb of sequences 5' to the PSMA transcription start site and tested their ability to provide increased reporter gene expression; no increased activity was seen relative to the 1 kb promoter region.

A strategy was developed to allow screening of DNA fragments for their ability to enhance transcription directed by the 1 kb proximal promoter region of the PSMA gene. The 1 kb promoter was cloned in front of the Green Fluorescent Protein (GFP) gene in the plasmid vector pPSMentrap shown in Figure 1. Upstream of the promoter was inserted a polylinker region containing sites for cloning candidate fragments.

pPSMentrap contains the following elements: a polylinker containing restriction sites for the enzymes Kpn1, HindIII, SalI, MfeI, NsiI, BglI, NheI and SpeI, the PSMA promoter region stretching from base 1386 base 2560 (XbaI site) of the PSMA sequence (Genbank Accession No. AF007544), a chimeric intron as contained in the pC1 vector (Promega), the GFP gene, the 3' end polyadenylation signal from the bovine growth hormone gene and the plasmid backbone (including ampicillin resistance gene and origin of replication) from the pC1 vector.

A library of DNA sequences was prepared by digesting the bacteriophage P1 cosmid P1-683 which contains the 5' half and upstream flanking sequence of the PSMA gene (40). Cosmid DNA was digested for various of times with the

enzyme Tsp509I which cuts at AATT sites generating a range of partial digestion products. These were separated by agarose gel electrophoresis and fragments in the size range 1 to 2 kb recovered and cloned into the MfeI site of the pPSMentrap vector. A library of about 600 individual clones was picked. Clones were grouped into 12 pools of 49 and DNA prepared from each pool using Qiagen columns and protocols. DNA (2.5 mg) from each pool was transfected into LNCaP cells in 3.5 cm dishes as previously described (1). After 48 to 72 hours, cell cultures were examined under a UV fluorescence microscope to identify any fluorescing cells. Positive pools were split into 7 by 7 matrices and DNA preparations made from the 7 clones in each row and each column. The transfections were repeated to identify positive sub-pools. Clones at the intersections of positive rows and columns were further screened individually to verify the expression of GFP. The three clones giving the strongest signals, #1, #3 and #4 were taken for further analysis.

Example 2

Location and sequence analysis of enhancing fragments

The inserts from the clones were re-cloned into pBluescriptSK+ (pBKSEn3 and pBKSEn4) and the sequences of their ends determined. All clones were found to originate from the third intron of the PSMA gene as shown in Figure 2. The positions of both ends of clones #3 and #4 were identified as shown. The inserts in clones #3 and #4 were aligned in opposite orientations relative to the PSM promoter in the pPSMentrap vector as shown in Figure 3. The clones share a common overlapping sequence of 1044 bp and extend in total over 2,530 bp. The third clone, #1, derived from the same region, one end being 6 bp upstream of the end of clone #4 and it also contained the SpeI and HinDIII sites contained in the region common to clones #3 and #4. It had, however, undergone some rearrangement on cloning and has not been further studied.

Example 3

Function of PSMA enhancer region

The activity of the PSMA enhancer region was first identified by visual inspection of fluorescence intensity of cells transfected with clones carrying PSMA gene inserts upstream of the PSM promoter. In these preliminary experiments it was also noted that the enhancer (clone #4) did not appear to

function in the bladder cell line BL13 (not shown). In order to provide for quantitative determination of promoter and enhancer function, enhancers #3 and #4 (hereafter designated En3 and En4) in combination with the PSM 1kb promoter were re-cloned into two different gene expression reporter systems.

Example 4

Expression assayed in the pCAT3SAT system

The pCAT3SAT vector contains a modified bacterial chloramphenicol acetyl transferase reporter gene for determining promoter activity and a reference reporter gene, serine acetyl transferase, under the control of the RSV promoter in order to standardise CAT expression for transfection efficiency. It was prepared by cloning the serine acetyl transferase reporter gene from the pCATSAT plasmid (1) as a SalI/BamHI fragment into BamHI, SalI cut pCAT3 vector (Promega). Constructs, pPSM1k-C3S and pEn4PSM1k-C3S, containing the PSM promoter with or without the PSM enhancer fragment 4 (En4) were prepared by cloning the PSM enhancer/promoter fragments as SalI/PstI fragments from the pPSMentrap vector into pCAT3SAT cut at the XhoI and PstI sites in the polylinker upstream of the CAT gene (Figure 4). A control construct containing the RSV promoter, pRSV-C3S, was also prepared by blunt end ligation of a NaeI to SacI fragment from pCATSAT (1) into the NheI site of pCAT3SAT (Figure 4). Cell lines were transfected with the different constructs and CAT and SAT activities determined after 48 h as described (1). The normalised expression data are shown in Figure 5.

In LNCaP cells an enhancement of expression of approximately 50 fold (from 0.33% to 15.7% of the activity of the RSV promoter) was seen when the En4 fragment was present upstream of the 1 kb PSM promoter. This expression showed a high level of specificity for LNCaP cells that express PSMA. Another prostate cell line, PC3, showed very low levels of expression from the PSM promoter either in the presence or absence of the enhancer. No expression above background was seen for three non-prostate cell lines (MCF-7, a breast cancer line, human embryonic kidney cells (HEK293) and the liver line HepG2). Low and variable expression was seen in a second breast cancer cell line T47-D2, with the enhancer/promoter construct showing about 10% of the activity seen in LNCaP cells.

Example 5

Expression assayed in the luciferase pGL3 system

Because of the low activity of the PSM 1kb promoter in the CAT assay system, promoter and enhancer sequences were cloned into the pGL3 vector (Promega) which contains the luciferase reporter gene. The structure of the clones is shown in Figure 6. pPSM1k-GL3 and pEn4PSM1k-GL3 were prepared by cloning KpnI to XbaI fragments from pPSM1k-C3S and pEn4PSM1k-C3S respectively into pGL3 cut with KpnI and NheI. pEn3PSM1k-GL3 was prepared by cloning the KpnI to NheI enhancer fragment of pEn3PSMentrap into pEn4PSM1k-GL3 cut with KpnI and NheI. To assay activity, mixtures of each pGL3 construct and the reference plasmid pRSVCAT (1) were transfected into a variety of cell lines by standard procedures as described previously (1). DNA concentrations were determined by image analysis of ethidium bromide stained gels and master mixes prepared in the ratio of 1.5 μg of pGL3 construct to 1 μg of pRSVCAT. The same master mixes were used for transfections into all cell lines. Cells were transfected with 2.5 μg of DNA mixes using standard procedures (1) and expression assayed after 48 hr. Extracts were prepared and luciferase activity determined using the Luciferase Assay System (Promega). CAT activities were determined as previously described. Luciferase expression levels were standardised with respect to the pRSVCAT reference plasmid and then standardised activities expressed as a proportion of that of pRSV-GL3/pRSVCAT (Figure 7).

In LNCaP cells expression from the PSM 1k promoter was strongly enhanced by both En3 and En4 enhancer sequences (about 260 fold) with expression levels directed by pEn3PSM1k and pEn4PSM1k being 15 and 15.7% that of the RSV promoter. In the non-PSMA-expressing prostate cell line PC3 a low level of enhancement (3.7 and 5.2 fold for En3 and En4 respectively) was seen, while there was no enhancer function in the other non-expressing prostate line, DU145. For a range of non-prostate cell lines tested, HepG2 liver cells, MRC5 primary lung fibroblasts, BL13 bladder carcinoma and human embryonic kidney HEK293 cells, essentially no activity was seen for the PSMA enhancer/promoter or promoter alone constructs. Activity is thus highly specific for the expressing prostate cell line LNCaP with partial enhancer function in one non-expressing prostate cell line PC-3.

Example 6

Characterisation of the enhancer element

To determine the extent of sequences required to provide enhancer activity a construct was prepared that contained all the sequences encompassed by clones En3 and En4 as well as constructs containing the overlapping region present in both cones (see Figure 6). pEn3+4PSM1k-GL3 was prepared by cloning a KpnI to NdeI restriction fragment from pBKSEn3 into pEn4PSM1k-GL3 cut with KpnI and NdeI. Clone pOverlapen3/4a was prepared by cloning the SalI to HinDIII fragment from pEn3PSMentrap into pBluescriptSK+, subsequently cloning the HindIII fragment from pEn4PSMentrap into the HinDIII site of the intermediate vector and verifying that it was in the correct orientation. The overlapping enhancer fragment was then cloned as a KpnI to EcoRI fragment in front of the PSM 1kb promoter in pPSM1k- GL3 cut with KpnI and EcoRI. A construct with the overlapping region in the opposite orientation relative to the PSM promoter was likewise prepared by first cloning the SalI to HinDIII fragment from pEn4PSMentrap into pBluescriptSK+ followed by the HinDIII fragment from pEn3PSMentrap and then cloning the overlap region in front of the PSM promoter as a KpnI to EcoRI fragment.

The effectiveness of these constructs was compared with that of the PSM1k promoter alone and the En4/PSM1k promoter by transfection (as above) into LNCaP cells. Clones containing either orientation of the overlap region gave rise to expression levels similar to those containing En 4 sequences. The construct containing the whole region encompassed by enhancers 3 and 4, however, gave significantly stronger expression. The level of expression was about half that of the RSV promoter.

Example 7

PSMA enhancer action on other promoters

The properties of the enhancer were further assessed by linking it to other promoters, both those active primarily in prostate cells, PSA and probasin, and a non-tissue-specific promoter, that of the herpesvirus thymidine kinase gene (TK). The structures of these promoter regions are shown in Figure 8. For the PSA and probasin constructs the enhancer region, En4, was cloned as an NheI fragment from the pEn4PSM1k-C3S plasmid into the XbaI-cut plasmids pPSA630 CATSAT and pPb430 CATSAT respectively (by partial

digestion with XbaI for the probasin construct). pPSA630CATSAT and pPb430 CATSAT have been described previously (1). The plasmid pTKCATSAT.1 was prepared by cloning the TK promoter region, bases −101 to +59, as a SalI to XhoI fragment into the SalI-cut vector pCATSAT.1 (1)[pCATSAT.1 is a derivative of pCATSAT (1) in which SalI, PstI and XhoI sites present upstream of the RSV promoter were removed or destroyed by XhoI and partial SalI digestion and religation]. pEn4TKCATSAT was prepared by cloning the SalI to BglII enhancer-containing fragment from pEn4PSMentrap into pTKCATSAT.1 cut with SalI and partially cut with BamHI.

All six plasmids were transfected into a number of cell lines and CAT and SAT reporter gene expression determined as described (1). Expression levels were standardised against that of the RSV promoter determined by transfection of a standard mixture of pRSVCAT and pRSVSAT plasmids as described (1). Results are shown in Figure 9a & b.

In LNCaP cells strong enhancement of the PSA, probasin and TK promoters was seen, with that for probasin being strongest. Levels of expression for all enhancer constructs were 2 to 3 times that of the RSV promoter. Since all promoters achieved similar levels of expression in the presence of the enhancer the "fold-enhancement" shown probably reflects differences in the level of non-enhanced expression from the different promoters,

In PC3 prostate cells, which do not express PSMA, much reduced enhancement was seen, being 5 to 16 fold for the different promoters. This is similar to the results seen when the enhancer was joined with its own PSM promoter. Thus it appears that PC3 cells contain some factors that can interact with the PSM enhancer to activate transcription, but lack others, or do not have sufficient levels, to enable full enhancer function as is seen in LNCaP cells.

For the non-prostate cell lines, no enhancement was seen in HepG2 liver or BL13 bladder cells. Enhancement was seen in the embryonic kidney HEK293 cells. Low level enhancement (1.4, 1.5 fold) was seen for the PSA and TK promoters, while there was a stronger 9 fold enhancement of the probasin promoter. No enhancement by En4 of its homologous PSM promoter was seen in HEK293 cells (Figure 7). Since the proximal kidney tubules are a site of low level PSMA expression, the expression seen in HEK293 cells may be biologically meaningful.

Example 8

PSM Enhancer Function Does not Require Androgens

The androgen requirement for activity of the PSM enhancer (En4) was studied when it was linked to two highly androgen-inducible promoters, those of the probasin and PSA genes and one constitutive promoter, TK. LNCaP cells were transfected with plasmid constructs using media that had been charcoal stripped to remove androgens. Cells were maintained in androgen-free medium or incubated in the presence of the non-metabolizable androgen analogue, R1881 added to 0.28 nM (1).For all promoters strong enhancement of expression was seen whether or not androgen was present in the medium. However, for all three constructs containing the PSM enhancer the level of expression actually decreased upon androgen addition. This suggests that the enhancer may contain sequences mediating the observed androgen-suppression of the endogenous PSMA gene.

Example 9

Sequences required for enhancer function

In order to determine what sequence regions were critical for enhancer function a series of constructs were prepared in which different fragments from the PSME region were placed in front of the PSM promoter in the pPSM1k-GL3 plasmid. The sequences included in each construct are shown in the table below. The orientation of the enhancer sequences relative to the promoter is indicated as either F (forward, as for pEn4PSM1k-GL3) or R (reverse, as for pEn3PSM1k-GL3). Activity of these constructs was assayed following transfection into LNCaP cells along with the pRSVCAT control plasmid. Extracts were prepared and assayed 48 hr after transfection, luciferase activity normalised using the activity of the co-transfected pRSVCAT plasmid and expressed relative to that of pRSV-GL3 (Table below).

Construct	Enhancer sequences	Activity in LNCaP cells (% RSV)
pPSM1k-GL3		0.2
pEn4PSM1k-GL3	14760 - 16575 F	16.0
pEn3PSM1k-GL3	14045 - 15804 R	15.7

pEn3+4PSM1k-GL3	14045 - 16575 F	39
pEn3/4aPSM1k-GL3	14760 - 15804 F	25
pEn3/4bPSM1k-GL3	15804 - 14760 R	21
pEn4SalHindIIIPSM1k-GL3	14760 - 15374 F	20
pEn3SalHindIIIPSM1k-GL3	15804 - 15369 R	0.1
pEnO2/770SpeIPSM1k-GL3	14760 - 15530 F	24
pEnO2/2/592NsiIPSM1k-GL3	14760 - 15352 F	22
pEnO2/445MscIPSM1k-GL3	14760 - 15205 F	18
pEnO2/331SmaIPSM1k-GL3	14760 - 15091 F	26
pEnO2/168NdeIPSM1k-GL3	14760 - 14930 F	6
pEnO1/722SmaIPSM1k-GL3	15092 - 15804 R	0.3
pEnO1/886NdeIIPSM1k-GL3	14925 - 15804 R	0.4

These data indicate that most of the enhancer activity is contained within the 331 bp region encompassing bases 14760 to 15091. This region shows similar activity (26% that of RSV) to the En3 and En4 clones and to the approximately 1kb region shared between them. Deletion from the 1 kb overlap region of either the left half or the entire 331 bp region (constructs pEnO1/722SmaIPSM1k-GL3 and pEnO1/886NdeIIPSM1k-GL3) eliminates enhancer activity, showing that this region is essential for activity. Elimination of the right half of the 331 bp region, leaving just 170 bp covering bases 14760 to 14930, leads to a marked reduction in activity.

Thus bases 14760 to 14930 are essential for PSME function, but sequences extending from 14760 to 15091, provide for much stronger enhancer activity. The sequence of the region is shown in Figure 11.

Example 10

PSME core enhancer region retains cell-type specificity

Experiments were carried out on the 331 bp core region of the PSME that provides for enhancer function (bases 14760 to 15091) to determine whether this region retained its cell-type specificity. The activity of plasmids pPSM1k-GL3, pEn02/331SmaIPSM1k-GL3 and pRSV-GL3 was assayed after transfection into a number of cell lines (Table below). Plasmids were co-transfected with an internal control pRSVCAT plasmid, extracts prepared and assayed 48 h after

transfection. Luciferase activities were normalised using the activity of the pRSVCAT plasmid and are expressed relative to that of pRSV-GL3.

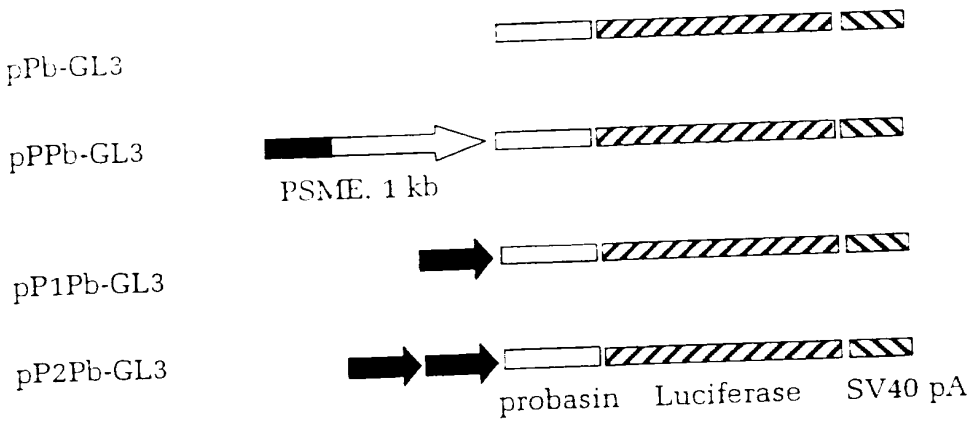
Construct	Activity Relative to the RSV promoter (%)				
	PC-3	DU145	MCF7	MRC5	HepG2
pPSM1k-GL3	0.45	0.21	0.12	0.032	0.033
pEn02.331SmaIPSM1k-GL3	1.70	0.13	0.14	0.048	0.022

As for the longer enhancer fragments, partial enhancer activity was seen in the PC-3 prostate cancer cell line that does not express PSMA. For the other non-PSMA expressing prostate cell line, DU145, no enhancement of basal promoter activity was seen. Likewise the 331 bp PSME core region is not functional in three non-prostate cell lines. The core region thus retains the specificity of the PSME.

Example 11

Tandem enhancer sequences provide for greater activity

A series of constructs were prepared in which the probasin promoter, with or without PSM enhancer fragments was subcloned in front of the luciferase reporter gene in the pGL3 vector. The structure of the constructs is shown below. The 430 bp probasin promoter fragment has been described previously (1) and was re-cloned from the pPB-CS plasmid (see Figure 8). pPPb-GL3 contains the 1 kb overlapping enhancer region (bases 14760 to 15804). pP1Pb-GL3 and pP2PPb-GL3 contain one or two copies respectively of the 331 bp enhancer region (bases 14760 to 15091). All enhancer sequences are in the forward orientation.



The constructs were transfected, along with an RSVCAT control plasmid, into LNCaP, HEK293 or MCF-7 cells and expression measured in cell extracts prepared after 48 h incubation. Transfections were done in androgen-depleted media and luciferase activity corrected using the co-transfected RSVCAT internal control.

Relative Luciferase Activity

	LNCaP	HEK293	MCF-7
pPb-GL3	1.45	2.36	0.36
pPPb-GL-3	246	2.17	1.09
pP1Pb-GL-3	346	3.2	0.73
pP2Pb-GL-3	798	1.8	5.75
pRSV-GL-3	318	277	107

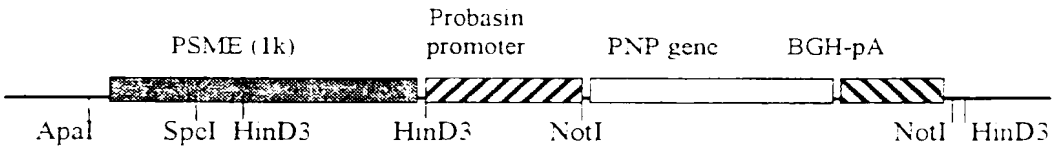
Greatest expression in LNCaP cells is seen with the double enhancer construct, being 2 to 3 times greater than those constructs with a single copy of the enhancer. Specificity of expression is largely maintained in these transfection studies, though the pP2Pb-GL3 construct shows an elevated level of expression in MCF-7 cells.

Example 12

Enhancer function in a viral backbone

The properties of the PSME combined with the probasin promoter (its high activity and specificity and limited responsiveness to androgen levels) are particularly suitable for directing prostate-specific gene expression in gene therapy applications.

The *E. coli* purine nucleoside phosphorylase (PNP) gene in combination with the pro-drugs fludarabine or 6-methylpurine 2-deoxyriboside (6MPDR) can be used to deliver enzyme pro-drug therapy (41). An expression cassette was prepared in the pGEM11 plasmid in which the PNP gene was placed under the control of the 1 kb PSME region (bases 14760 to 15804 in reverse orientation) adjacent to the 430 bp probasin promoter. A map of this construct (pPPP (for Psm Probasin PNP)) is shown below. The cassette in pGEM11 was partially sequenced to confirm its structure



The expression cassette was subcloned by cutting with ApaI and NotI (partial digest for NotI) and inserting into ApaI/NotI cut ovine adenovirus (OAV) vectors (42). The expression cassette was inserted into two separate sites in the OAV plasmid. One isolate was prepared by cloning into OAV200 cut with ApaI and NotI (Site 1) to give clone pOAV223. In the other isolate, pOAV623, the cassette was cloned in an alternate site (Site 3) of the plasmid pOAV600 (42). Plasmid DNA was transfected into CSL503 cells as described (43) and viruses OAV223 and 623 recovered.

OAV223, OAV623 and two other viruses OAV220 and OAV222, that are equivalent to OAV223 except that the PNP gene is under the control of the RSV and CMV promoters respectively, were used to infect a variety of cell types as shown in Figure 12. Cells were infected with the different viruses at a multiplicity of infection of 10^3 opu/cell and PNP expression measured after 4 days (44). For each cell type an amount of lysate was used such that PNP

expression from the most strongly expressing virus fell within the linear range of the assay. Thus, the absolute amount of PNP activity cannot be compared between cell lines but ratios of expression can be compared.

The data presented in Figure 12 show that in the context of the viral backbone and OAV infection strong specificity of gene expression is maintained. Highest activity is seen from OAV623, then OAV223, being greater than that of the RSV promoter in LNCaP and LN3 prostate cancer cells. In all the non-prostate cell lines the RSV promoter (OAV220) provides strongest expression. The differential specificity of the PSME/Pb promoter versus the RSV promoter for prostate compared to non-prostate cells ranges from expression about 15 fold for HEK293 and MCF-7 through to 200 fold for MRC-5). Thus, in some cell types specificity is reduced in the OAV context but it is still substantial. In the following example retention of cell specificity of the PSME in combination with its own PSM promoter is also demonstrated when carried by a human adenovirus Type 5.

Example 13

Enhancer function in human umbilical artery cells

PSMA has been shown to be expressed in the neovasculature of a range of tumour types, but not in normal vasculature. We have determined, using reverse transcriptase PCR, that PSMA is expressed in endothelial cells derived from the human umbilical artery (HUAECs) (data not shown). Other genes that are up-regulated in tumour vasculature are also expressed in HUAECs and related human umbilical vein cells (HUVECs), eg endoglin (45). Function of PSM regulatory sequences was therefore examined in these cells. The activity of the PSME coupled to the PSM 1 kb promoter was evaluated using a replication-defective adenovirus, human adenovirus Type 5, into which the expression cassette from the pPSMentrap vector with the En4 insert had been inserted. The virus, Ad525, carries the GFP gene with bovine growth hormone 3' polyadenylation sequences under the transcriptional control of PSME En4 sequences coupled to the PSM 1 kb promoter. A control virus, Ad526, in which the GFP gene was under the control of the ubiquitously-active EF-1 promoter was also used.

HUAECs and HUVECs were dissociated from umbilical arteries and cultured as described by Underwood and Bean (46) except that tissue culture dishes were coated with bovine, rather than chicken, fibronectin. HUACs,

HUVECs, LNCaP and control human lung fibroblast MRC-5 cells were plated at 4×10^4 cells per chamber in fibronectin-coated microscope slide chambers. The following day they were infected with 5×10^8 optical particle units per chamber of either Ad525 or Ad526. Expression of the GFP gene was monitored by fluorescence microscopy 3 days after infection for the control Ad526 virus and after 6 days for the PSME driven Ad525.

Expression from the control virus (EIF, OAV526) was strong in all cell types. For the En4PSMGFP virus, clear expression was seen in HUAECs and LNCaP cells, weaker expression in HUVECs, but no expression could be detected in MRC-5 cells. The combination of PSME and the PSM promoter is thus able to specifically drive gene expression in these arterial cells that express the endogenous PSM gene and should prove useful in directing expression to tumour vasculature.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

REFERENCES

1. Brookes, D. E., D. Zandvliet, F. Watt, P. J. Russell and P. L. Molloy (1998) relative activity and specificity of promoters from prostate-expressed genes. The Prostate **35**: 18-26

2. Cleutjens, K. B. J. M., C. C. E. M. Vaneekelen, H. A. G. M. Vanderkorput, A. O. Brinkmann and J. Trapman. (1996). "Two androgen response regions cooperate in steroid hormone regulated activity of the prostate-specific antigen promoter." J Biol Chem. **271**(11): 6379-6388.

3. Riegman, P. H. J., R. J. Vliestra, J. A. G. M. van der Korput, A. O. Brinkmann and J. Trapman. (1991). "The promoter of the prostate-specific antigen gene contains a functional androgen responsive element." Molec. Endocrinology. **5**(12): 1921-1930.

4. Murtha, P., D. J. Tindall and C. Y. F. Young. (1993). "Androgen induction of a human Prostate-Specific kallikrein, hKLK2 - characterization of an androgen response element in the 5' promoter region of the gene." Biochemistry. **32**(25): 6459-6464.

5. Claessens, F., N. K. Rushmere, P. Davies, L. Celis, B. Peeters and W. A. Rombauts. (1990). "Sequence-specific binding of androgen-receptor complexes to prostatic binding protein genes." Mol. Cell. Endocrinol. **74**: 203-212.

6. Rushmere, N. K., M. G. Parker and P. Davies. (1987). "Androgen-receptor binding regions of an androgen responsive gene." Mol. Cell. Endocrinol. **51**: 259-265

7. Kasper, S., P. S. Rennie, N. Bruchovsky, P. C. Sheppard, H. Cheng, L. Lin, R. P. C. Shiu, R. Snoek and R. J. Matusik. (1994). "Cooperative binding of androgen receptors to two DNA sequences is required for androgen induction of the probasin gene." J. Biol. Chem. **269**(50): 31763-31769.

8. Rennie, P. S., N. Bruchovsky, K. J. Leco, P. C. Sheppard, S. A. McQueen, H. Cheng, R. Snoek, A. Hamel, M. E. Bock, B. S. MacDonald, B. E. Nickel, C. Chang, S. Liao, P. A. Cattini and R. J. Matusik. (1993). "Characterization of two cis-acting DNA elements involved in the regulation of the probasin gene." Molec. Endocrinol. **7**(1): 23-36.

9. Virkkunen, P., P. Hedberg, J. J. Palvimo, E. Birr, K. Porvari, M. Ruokonen, P. Taavitsainen, O. A. Jänne and P. Vihko. (1994). "Structural comparison of human and rat prostate-specific acid phosphatase genes and their promoters:

identification of putative androgen response elements." Biochem. and Biophys. Res. Commun. **202**(1): 49-57.

10. Celis, L., F. Claessens, B. Peeters, W. Heyns, G. Verhoeven and W. Rombauts. (1993). "Proteins interacting with an androgen-responsive unit in the C3(1) gene intron." Mol. Cell. Endocrinol. **94**: 165-172.

11. Ho, K. C., K. B. Marschke, J. A. Tan, S. G. A. Power, E. M. Wilson and F. S. French. (1993). "A complex response element in intron-1 of the Androgen-Regulated 20-kDa protein gene displays cell Type-Dependent androgen receptor specificity." J Biol Chem. **268**(36): 27226-27235.

12. Zhang, Y.-L., M. G. Parker and O. Bakker. (1990). "Tissue-specific differences in the binding of nuclear proteins to a CCAAT motif in the promoter of the androgen-regulated C3 gene." Molec. Endocrinol. **4**(8): 1219-1225.

13. Allison, J., Y.-L. Zhang and M. G. Parker. (1989). "Tissue-specific and hormonal regulation of the gene for rat prostatic steroid-binding protein in transgenic mice." Mol. Cell. Biol. **9**: 2254-2257.

14. Maroulakou, I. G., M. Anver, L. Garrett and J. E. Green. (1994). "Prostate and mammary adenocarcinoma in transgenic mice carrying a rat c3(1) simian virus 40 large tumor antigen fusion gene." Proc Natl Acad Sci USA. **91**(23): 11236-11240.

15. Greenberg, N. M., F. J. Demayo, P. C. Sheppard, R. Barrios, R. Lebovitz, M. Finegold, R. Angelopoulou, J. G. Dodd, M. L. Duckworth, J. M. Rosen and R. J. Matusik. (1994). "The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice." Mol Endocrinol. **8**(2): 230-239.

16. Matusik, R. J. Isolated DNA mol. contg. an androgen responsive element comprising a 5'-flanking region of the rat probasin gene, used to generate transgenic animals and for human prostate cancer therapy. International publication no. WO 94/03594

17. Greenberg, N. M., F. Demayo, M. J. Finegold, D. Medina, W. D Tilley, J. O. Aspinall, G. R. Cunha, A. A. Donjacour, R. J. Matusik and J. M. Rosen. (1995). "Prostate cancer in a transgenic mouse." Proc Natl Acad Sci USA. **92**(8): 3439-3443.

18. Gingrich, J. R., R. J. Barrios, M. W. Kattan, H. S. Nahm, M. J. Finegold and N. M. Greenberg (1997) Androgen independent prostate cancer progression in the TRAMP model. Cancer Res. **57**: 4687-4691.

19. Schaffner, D. L., R. Barrios, M. R. Shaker, S. Rajagopalan, S. L. Huang, D. J. Tindall, C. Y. F. Young, P. A. Overbeek, R. M. Lebovitz and M. W. Lieberman. (1995). "Transgenic mice carrying a PSArasT24 hybrid gene develop salivary gland and gastrointestinal tract neoplasms." Lab Invest. **72**(3): 283-290.

20. Belldegrun, A. S. and S. Pang. "Nucleic acid contg. prostate specific antigen promoter useful for prodn. of heterologous protein, or for gene therapy of prostatic cancer." : International publicationno. WO 96/14875

21. Pang, S., S. Taneja, K. Dardashti, P. Cohan, R. Kaboo, M. Sokoloff, C.-L. Tso, J. B. Dekernion and A. S. Belldegrun. (1995). "Prostate tissue specificity of the prostate-specific antigen promoter isolated from a patient with prostate cancer." Hum. Gene Ther. **6**: 1417-1426.

22. Henderson, D. R. Transcriptional regulator specific for cells expressing prostate specific antigen used to express toxins, immunostimulants or anti-sense cpds., for treatment and prevention of prostatic cancer or hypertrophy. Internation publication no. WO 95/19434

23. Schuur, E. R., G. A. Henderson, L. A. Kmetec, J. D. Miller, H. G. Lamparski and D. R. Henderson. (1996). "Prostate-specific antigen expression is regulated by an upstream enhancer." J Biol Chem. **271**(12): 7043-7051.

24. Rodriguez, R., E. R. Schuur, H. Y. Lim, G. A. Henderson, J. W. Simons and D. R. Henderson (1997) Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res.**57**: 2559-2563.

25. Horoszewicz, J. S., E. Kawinski and G. P. Murphy (1987) Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostate cancer patients. Anticancer Res. **7**: 927-936.

26. Troyer, J. K., Q. Feng, M. L. Beckett and G. L. Wright Jr. (1995) Biochemical characterization and mapping of the 7E11-C5.3 epitope of the prostate specific membrane antigen. Urol. Oncol. **1**: 29-37.

27. Troyer, J. K., M. L. Beckett and G. L. Wright Jr. (1997) Location of the prostate specific membrane antigen in the LNCaP prostate carcinoma cell line. The Prostate **30**: 232-242.

28. Liu, H., P. Moy, S. Kim, Y. Xia, A. Rajasekaran, V. Navarro, B. Knudsen and N. H. Bander (1997) Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. Cancer Res. **57**: 3629-3634.

29. Murphy, G. P., W. J. Tino, E. H. Holmes, A. L. Boynton, S. J. Erikson, V. A. Bowes, R. J. Barren, B. A. Tjoa, S. L. Misrock, H. Ragde and G. M. Kenny (1996) Measurement of prostate-specific membrane antigen in he serum with a new antibody. The Prostate **28**: 266-271.

30. Israeli, R. S., C. T. Powell, W. R. Fair and W. D. Heston (1993) Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen. Cancer Res. **53**: 227-230.

31. Su, S. L., I. P. Huang W. R. Fair and W. D. W. Heston (1995) Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression. Cancer Res. **55**: 1441-1443.

32. Pinto, J. T., B. P. Suffoletto, T. M. Berzin, C. H. Qiao, S. Lin, W. P. Tong, F. May, B. Mukherjee and W. D. W. Heston (1996) Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. Clinical Cancer Res. **2**: 1445-1451.

33. Carter, R. E., A. R. Feldman and J. T. Coyle (1996) Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. Proc. Natl. Acad. Sci. USA **93**: 749-753.

34. Wright, G. L., C. Haley, M. L. Beckett and P. F. Schellhammer (1995) Expression of prostate-specific membrane antigen in normal, benign, and malignant prostate tissues. Urol. Oncol. **1**: 16-28.

35. Lopes, D., W. L. Davis, M. J. Rosenstraus, Uveges, A. J. and S.C. Gilman (1990) Immunohistochemical and pharmacokinetic characterization of the site-specific immunoconjugate CYT-356 derived from the antiprostate monoclonal antibody 7E11-C5. Cancer Res. **50**: 6423-6429.

36. Silver, D. A., I. Pellicer, W. R. Fair, W. D. W. Heston and C. Cordon-Cardo (1997) Prostate-specific membrane antigen expression in normal and malignant tissues. Clin. Cancer Res. **3**: 81-85.

37. Israeli, R. S., C. T. Powell, J. G. Corr, W. R. Fair and W. D. W. Heston (1994) Expression of the prostate-specific membrane antigen. Cancer Res. **54**: 1807-1811.

38. Wright, G. L. Jr., M. Grob, C. Haley, K. Grossman, K. Newhall, D. Petrylak, J. Troyer, A. Konchuba, P. F. Schellhammer and R. Moriarty (1996) Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. Urology **48**: 326-334.

39. Fair, W. R., R. S. Israeli and W. D. W. Heston (1997) Prostate-specific membrane antigen. The Prostate 32: 140-148.

40. O'Keefe, D. S., S. L. Su, D. J. Bacich, Y. Horiguchi, Y. Luo, C. T. Powell, D. Zandvliet, P. J. Russell, P. L. Molloy, N. J. Nowak, T. B. Shows, C. Mullins, R. A. Vonder Haar, W. R. Fair and W. D. W. Heston (1998) Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. Biochim et Biophys. Acta 1443: 113-127.

41. Lockett, L.J., Molloy, P.L., Russell, P.J. and Both, G.W. (1997) Relative Efficiency Of Tumour Cell Killing In Vitro By Two Enzyme-Prodrug Systems Delivered By Identical Adenovirus Vectors. Clinical Cancer Res. 3:2075-2080.

42. Xu ZZ, Hyatt A, Boyle DB, Both GW. (1997) Construction of ovine adenovirus recombinants by gene insertion or deletion of related terminal region sequences. Virology 230:62-71.

43. Vrati S, Macavoy ES, Xu ZZ, Smole C, Boyle DB, Both GW. (1996) Construction and transfection of ovine adenovirus genomic clones to rescue modified viruses.Virology 220:200-203.

44. Martiniello-Wilks, R., Garcia-Aragon, J., Daja, M., Russell, P., Both, G.W., Molloy, P.L., Lockett, L.J. and Russell, P.J. (1998) Human Gene Therapy 9: 99-106.

45. Graulich W., Nettlebeck, D.M., Fischer D., Kissell T and R. Muller (1999) Cell type specificity of the human endoglin promoter. Gene 227; 55-62.

46. Underwood, P.A. and P.A. Bean (1996) The effect of vitronectin and other extracellular matrix molecules on endothelial expansion and plasminogen activation. Cells and Materials 6: 193-207.